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Role of Chemical Inducers in Larval Metamorphosis of Queen Conch, *Strombus gigas* Linnaeus: Relationship to Other Marine Invertebrate Systems

ANNE A. BOETTCHER* AND NANCY M. TARGETT

University of Delaware, Graduate College of Marine Studies, Lewes, Delaware 19958

Abstract. Chemical cues are important in the exogenous and endogenous control of metamorphosis in many marine invertebrate larvae. In the queen conch, *Strombus gigas* Linnaeus, larval metamorphosis is induced by low molecular weight compounds associated with dominant species of red algae found in conch nursery grounds; these species include the foliose rhodophyte *Laurencia poitei* (Lamouroux). The responses of conch larvae to the algal-associated cues are dependent on concentration and length of exposure, with the initial events of metamorphosis occurring within 10 min of treatment with an aqueous extract of *L. poitei.* The free amino acids valine and isoleucine mimic the effects of the natural inducer, and they may bind to and be recognized by the same sites on the larvae as the algal cues. Hydrogen peroxide, vanadate, and γ-aminobutyric acid (GABA), as well as elevated K^+ concentrations (*i.e.*, above ambient seawater levels), also induce larval metamorphosis. Acetylsalicylic acid decreases the responses of conch larvae to the algal-associated cues and to the free amino acids, but it has no effect on the induction triggered by hydrogen peroxide. The chemical induction of metamorphosis in conch larvae shares many general features with chemoreception in aquatic invertebrates. The natural inducers of metamorphosis, like the cues involved in olfactory responses in other marine organisms, are of low molecular weight and water soluble. In addition, the results of the experiments with hydrogen peroxide, vanadate, and GABA suggest that second messenger pathways are involved in conch metamorphosis.

Introduction

Queen conch, *Strombus gigas*, are marine benthic gastropods found in seagrass beds and sand flats throughout the tropical Atlantic (Randall, 1964; Brownell and Stevely, 1981). As juveniles, conch occur primarily in seagrass beds of medium shoot density and in surrounding sandy areas where they feed on macrophytes and macrophyte epibionts (Ray and Davis, 1989; Stoner and Sandt, 1991; Stoner and Waite, 1991; Wickland *et al.*, 1991; Sandt and Stoner, 1993; Stoner and Ray, 1993; Ray and Stoner, 1994; Stoner *et al.*, 1994). The mechanisms by which conch larvae find their nursery grounds and metamorphose to juveniles are not well understood, but recent work has shown that a variety of chemical cues associated with nursery-ground substrates induce queen conch metamorphosis (Davis, 1994; Davis and Stoner, 1994; Boettcher and Targett, 1996; Stoner *et al.*, 1996). The most consistent and effective inducers are of low molecular weight (less than 1 kDa), stable, water soluble, and associated with the red algal species *Laurencia poitei* and *Fosliella* sp. (Boettcher and Targett, 1996). Crude aqueous extracts of the rhodophytes and a low molecular weight fraction (less than 1 kDa) of those extracts induce larval metamorphosis at levels comparable to those induced by the intact algae (Boettcher and Targett, 1996). These results suggest that chemical cues used by conch larvae during metamorphosis share many of the characteristics of those used by adult aquatic invertebrates. Marine invertebrates are sensitive to compounds of low molecular size, including the small peptides and amino acids that in adults serve as important cues in feeding, habitat selection, and mating, and in larvae as natural inducers of settlement and metamorphosis (Burke, 1984, 1986; Carr, 1988; Carr *et al.*, 1989; Tegtmeyer and Rittschof, 1989;

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* Current address: Neuroscience Research Institute, University of California, Santa Barbara, California 93106.

Morse, 1990; Rittschof, 1990; Morse, 1992; Leitz *et al.*, 1994; Zimmer-Faust and Tamburri, 1994).

The effects of chemical cues on metamorphosis are ideally studied with queen conch larvae. Moreover, several research facilities and one commercial facility (Caicos Conch Farm, Turks and Caicos, British West Indies) are currently involved in the culture of queen conch. By providing a better understanding of the biology of this species, detailed studies of metamorphosis in *S. gigas* could lead to improvements in the culture of this valuable mollusc (Heyman *et al.*, 1989; Boettcher *et al.*, 1997). In turn, the commercial culture of queen conch makes large numbers of larvae available for research.

In this study, the responses of *S. gigas* larvae to chemical cues were further characterized: the concentration dependency and the optimal exposure time to aqueous extracts of *L. poitei* were determined; the order of behavioral and gross morphological changes that occur in conch larvae during metamorphosis were described; and the effects of low molecular weight compounds (*e.g.*, amino acids, ions, and neuroactive compounds) on conch larvae metamorphosis were established. The results of these experiments are discussed in terms of models that have been developed to explain the transduction of cues involved in invertebrate larval metamorphosis (Burke, 1983; Baloun and Morse, 1984; Coon *et al.*, 1985; Baxter and Morse, 1987; Bonar *et al.*, 1990; Freeman and Ridgeway, 1990; Beiras and Widdows, 1995; Clare *et al.*, 1995).

Materials and Methods

Collection of algae and preparation of extracts

Specimens of *Laurencia poitei* were collected off Pine Cay, Turks and Caicos, British West Indies, and the crude aqueous extract was prepared as described in Davis (1994) and Boettcher and Targett (1996). Briefly, the alga was chopped into small pieces and ground in seawater (0.6 g alga/ml seawater) with a mortar and pestle. The sample was centrifuged at low speed in a table-top centrifuge for 10 min so that the algal pieces would pellet out of solution. The supernatant was decanted and used in all metamorphosis assays.

For amino acid analysis, the algal extract was prepared in distilled water and fractionated with a 400-ml Amicon (Amicon Division, W.R. Grace, Danvers, MA) stirred cell with 76-mm membranes. Amicon Diaflo ultrafiltration membranes (YM1) were used to separate the extract into nominal molecular sizes of less than and greater than 1 kDa (Boettcher and Targett, 1996). The fraction containing molecules smaller than 1 kDa was hydrolyzed in 6 *M* hydrochloric acid, phenol, and trifluoroacetic acid and analyzed with a single-column Beckman 6300 autoanalyzer (Tsugita *et al.*, 1987). Hereinafter, we refer to this as the < 1 kDa component of the algal extract.

Metamorphosis assay procedures

All metamorphosis assays were conducted at the Caicos Conch Farm, Providenciales, Turks and Caicos, British West Indies, according to the methods described in Boettcher and Targett (1996). Competent *Strombus gigas* larvae (19–24 days post-hatch) were provided by the Caicos Conch Farm. Techniques for their culture were as described in Davis (1994). Metamorphosis assays were run as static, no-choice experiments in 500-ml polyethylene vessels containing either 300 ml of seawater or seawater to which the appropriate treatment had been added. Seawater used in all experiments was sterilized with ultraviolet light and filtered (10 μm). Experiments were conducted at the ambient temperature (28°–29°C) and salinity (*ca.* 39 ppt), and under natural light conditions (*ca.* 12 h light: 12 h dark). The pH of the seawater was adjusted to between 8 and 8.5 with NaOH when necessary. For each assay, five replicates per treatment were used with either 15 or 25 larvae per replicate, depending on the experiment. Unless otherwise noted, the exposure time for all treatments was 5 h, after which the larvae were transferred to a fresh volume of seawater. Percent metamorphosis was determined after 24 h, and was calculated as 100 × (total number of larvae metamorphosed/total number recovered) (Pearce and Scheibling, 1990). A larva was considered to have undergone metamorphosis when it lost its velar lobes and began to use its foot to crawl (Davis, 1994). Each assay included a positive control (an aqueous extract of *L. poitei* [0.01 g wet weight/ml seawater = 20 μl extract/ml seawater]) as a measure of larval competency and a negative control (seawater only) as a test for spontaneous metamorphosis.

Mean percentages of metamorphosis among or between treatments in each experiment were compared with either a Model 1 ANOVA and Tukey's multiple comparison test or Student's t test ($\alpha = 0.05$). Plots of residuals were examined to assure that the underlying assumptions of these tests were met. Treatments in which percent metamorphosis was equal to zero for all replicates were not included in the statistical analyses.

Metamorphosis assays with algal extracts

The effects of concentration and duration of treatment on the metamorphogenic actions of crude aqueous extracts of *L. poitei* were tested according to procedures described above. In the first experiment, larvae were treated for 5 h with 11 dilutions of the *L. poitei* extract, ranging from 0.8 to 67 μl extract/ml seawater. In the second experiment, the larvae were exposed to 20 μl extract/ml seawater for 0.5, 1, 2, 3, and 5 h. As in all experiments, percentages of metamorphosis were determined after 24 h.

In an additional experiment, behavioral and morphological changes occurring in the larvae in response to the algal extract were monitored. Larvae treated with 20 μl extract/ml seawater were monitored with a compound microscope every 20 min, from time (t) = 0 to t = 3 h.

Metamorphosis assays with free amino acids

The effects of 11 free amino acids, all L-isomers (valine, leucine, isoleucine, cysteine, glycine, serine, threonine, lysine, arginine, histidine, and glutamic acid), were tested in assays of larval metamorphosis. Two criteria were used in choosing the amino acids to be tested: amino acids with basic, acidic, and neutral side chains were included, and all, except cysteine, were present in hydrolyzed samples of the less than 1 kDa component of the algal extract. Concentrations of amino acids tested ranged from 1 μ*M* to 10 m*M*. The free amino acid isoleucine (10 μ*M*) was also presented in combination with serine, histidine, and lysine (each at 10 μ*M*), and in combination with valine (each at 50 μ*M*).

Metamorphosis assays with acetylsalicylic acid

The effects of acetylsalicylic acid (aspirin, 0.1–5 m*M*), were examined alone and in combination with the algal extract (20 μl extract/ml seawater); treatments also included acetylsalicylic acid (1 m*M*) in combination with isoleucine and valine (each at 100 μ*M*). The effect of salicylic acid (1 m*M*) on the induction of metamorphosis by the algal extract was also tested. The responses of the larvae to *N*-acetyl-L-valine and to valine (both at 100 μ*M*) were compared. In an additional experiment, conch larvae were first treated with acetylsalicylic acid (1 m*M*) in combination with isoleucine (100 μ*M*), valine (100 μ*M*), or the extract, then rinsed in filtered seawater, and finally retreated for 5 h with isoleucine, valine, or extract. As a further examination of the effects of acetylsalicylic acid, the acetylsalicylic acid treatment was combined with exposure to 3-isobutyl-1-methyl xanthine (IBMX). The specific treatments in this experiment were IBMX (0.1 m*M*) or extract alone; IBMX plus acetylsalicylic acid (1 m*M*); acetylsalicylic acid plus extract; a combination of IBMX, acetylsalicylic acid, and the algal extract; and acetylsalicylic acid alone.

Metamorphosis assays with ion manipulations and neuroactive compounds

The effects on metamorphosis of elevated ion concentrations and of neuroactive compounds were examined in a series of experiments. The concentrations used were based on tests with other marine invertebrates (Baloun and Morse, 1984; Yool *et al.*, 1986; Davis *et al.*, 1990; Pires and Hadfield, 1991; Ilan *et al.*, 1993; Beiras and Widdows, 1995). Elevated concentrations of K^+, Ca^{2+}, Na^+, and Mg^{2+} (respectively 20 m*M*, 60 m*M*, 60 m*M*, and 60 m*M* above ambient seawater levels) were tested individually for their ability to induce metamorphosis. Two additional experiments focused on the concentration dependence of the response to increased K^+ concentrations (5–30 m*M*).

The responses of the larvae to the neuroactive compounds 3,4-dihydroxyphenylalanine (DOPA, 1–100 μ*M*), epinephrine (EP, 1 μ*M*), γ-aminobutyric acid (GABA, 0.1–20 m*M*), and hydrogen peroxide (50 and 100 μ*M*) were then examined. In addition, we tested the effects of compounds known to block the larval metamorphosis of other marine invertebrates (4 acetamino-4′isothiostilbene-2,2′disulfonic acid [SITS, 10 and 50 μ*M*] and tetraethylammonium chloride [TEA, 100 and 500 μ*M*]) for their effect on metamorphosis induced by algal extract (20 μl extract/ml seawater), hydrogen peroxide (50 μ*M*), and elevated K^+ concentrations (20 m*M*). SITS is an inhibitor of anion transport, and TEA is a K^+ channel blocker.

The larval responses to hydrogen peroxide (50 μ*M*) and to sodium orthovanadate (1 and 2 m*M*) were also compared. The effects of bovine catalase (5 μg/ml seawater) and acetylsalicylic acid (1 m*M*) on metamorphosis induced by hydrogen peroxide were compared with their effects on metamorphosis induced by the algal extract (20 μl extract/ml seawater).

Results

Metamorphosis assays with algal extracts

The concentration-function relationship of the response of conch larvae to the extract appeared to be hyberbolic (Fig. 1). Concentrations of algal extract lower than 5 μl/ml seawater had no significant effect on conch metamorphosis (Fig. 1). At levels greater than 13 μl/ml seawater, metamorphosis reached a plateau at about 85%. Concentrations of five μl extract/ml seawater and 6.7 μl/ml seawater induced increasing levels of conch metamorphosis (10% ± 6.0% and 37% ± 12% respectively). These two responses were significantly different from one another and from all higher ones.

The response to algal extract (20 μl/ml seawater) increased with duration of exposure (Fig. 2). The responses to the longest exposures, 3 h (76% ± 14%) and 5 h (91% ± 10%), were not significantly different. Thus, the exposure required to produce the maximal response is between 3 and 5 h.

Within 10 min of exposure to 20 μl extract/ml seawater, the velar cilia were arrested, and the conch larvae sank to the bottom of the experimental containers. Although the edges of the velar lobes started to curl, the lobes remained expanded and the cilia resumed beating

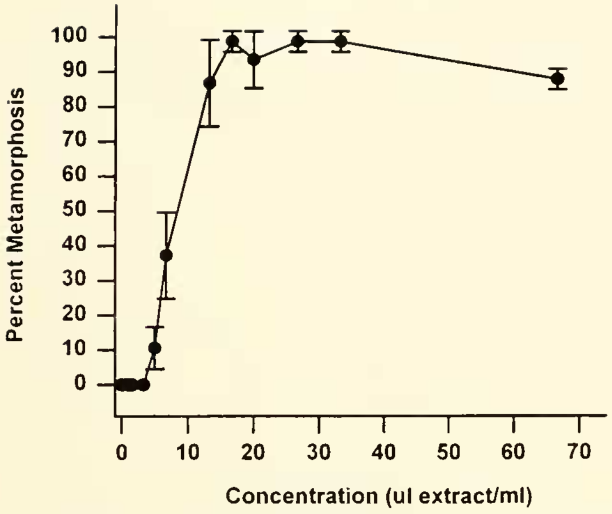

Figure 1. Percent metamorphosis of queen conch larvae in response to specific concentrations of *Laurencia poitei* extract. Points are means ± SD; *n* = 5.

after the larvae contacted the bottom. After 30 min, all larvae were on the bottom and their lobes showed increased curling; after 90 min, individual cilia and portions of the velar lobes began to drop off. Cilia on the isolated lobes continued to beat for at least 10 min. At 110 min, some of the larvae had completed metamorphosis. At this time, many still retained remnants of their velar lobes, but they had begun using the foot to crawl on the bottom of the containers. By 2 h, more than 50% of the larvae had undergone complete metamorphosis.

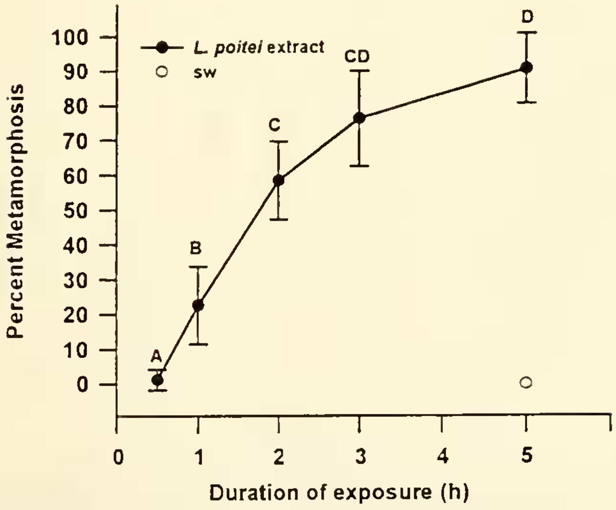

Figure 2. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control) for 5 h and an extract of *Laurencia poitei* (20 μl extract/ml seawater) for 0.5, 1, 2, 3, and 5 h. Points are means ± SD; *n* = 5. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

Amino acid composition of the algal extract

Of the 17 amino acids detected in the hydrolyzed <1 kDa component of the algal extract, the principal ones were glutamine/glutamate, glycine, alanine, and asparagine/aspartate (Table I).

Metamorphosis assays with amino acids

Of the 11 amino acids tested, only five (serine, histidine, leucine, isoleucine, and valine) induced significant levels of metamorphosis, and only two (isoleucine and valine) induced normal metamorphosis that was accompanied by normal behavior, as described above (Table II). Moreover, valine and isoleucine were present in the hydrolyzed extract at 6.6 and 4.2 μ*M* respectively (Table I).

At 50 μ*M* and 100 μ*M*, the levels of metamorphosis induced by isoleucine and valine were not significantly different from those induced by the algal extract, although at the lower concentration the response to isoleucine was significantly lower than that to valine. Isoleucine at 1 m*M* and 10 m*M*, the two highest concentrations tested, was again equipotent with the extract and showed no abnormal effects. At 10 μ*M*, isoleucine induced a high level of metamorphosis in one experiment (64% ± 18%), but a low level in a repeat of this experiment (14% ± 18%); and at the lowest concentration isoleucine had no effect (Table II), so the threshold is probably between 1 and 10 μ*M*.

Serine, histidine, and leucine also induced significant

Table I

Concentration of amino acids detected in the hydrolyzed less than 1 kDa fraction of an aqueous Laurencia poitei *extract*

Compound	Concentration (μ*M*)
Glutamine/Glutamate	46
Glycine	33
Alanine	19
Asparagine/Aspartate	18
Serine	14
Threonine	12
Homoserine	8.2
Valine	6.6
4-Hydroxyproline	6.2
Histidine	5.4
Leucine	4.4
Isoleucine	4.2
Hydroxylysine	3.6
Lysine	3.6
Arginine	3.0
Phenylalanine	2.8
Methionine	1.4

Only amino acids detected at concentrations greater than 1 μ*M* are listed.

Table II

Mean percent metamorphosis of Strombus gigas *larvae treated with specific concentrations of amino acids or an aqueous* Laurencia poitei *extract (20 μl/ml seawater)*

Concentration (μM)	Serine	Histidine	Leucine	Isoleucine	Valine	Extract
10,000	85 ± 9.9A*	dead	na	93 ± 6.5A	na	87 ± 4.7A
1,000	69 ± 14A	dead	na	86 ± 9.4A	na	65 ± 25A
100	0	88 ± 9.9A*,†	na	77 ± 5.8A	na	76 ± 9.2A
100	na	na	dead	61 ± 11A	68 ± 16A	82 ± 8.0A
100	na	na	na	64 ± 8.1A	85 ± 5.9B	84 ± 14AB
100	na	na	na	73 ± 13A	73 ± 6.7A	87 ± 9.4A
50	na	na	na	23 ± 11A	53 ± 10B	33 ± 19AB
10	0	4.0 ± 6.0A	na	14 ± 18A	na	96 ± 8.9B
10	na	na	68 ± 15A*	na	na	84 ± 8.9B
10	na	na	na	64 ± 18A	na	92 ± 5.6A
1	0	0	na	0	na	96 ± 8.9

Data presented as mean ± SD, $n = 5$; na indicates that the compound was not applied. Treatment results with the same letter adjacent to the mean are not significantly different at $P \leq 0.05$; each row represents a separate experiment. Unless otherwise indicated the metamorphosis responses were normal.

* Abnormal behavior (slow moving, not using foot to crawl).

† Many dead.

levels of metamorphosis, but the behavior of the larvae in response to these amino acids did not parallel that seen in response to the natural cue (Table II). The larvae tended to be slower moving and did not attach or begin crawling on the bottom as rapidly as with the algal inducer, isoleucine, or valine, if at all. Glutamic acid, arginine, threonine, and cysteine did not induce significant levels of metamorphosis at any of the concentrations tested. At 100 μM, cysteine was toxic to the larvae. Glycine induced low levels of metamorphosis (20%–30%) at concentrations between 100 μM and 1 mM. Lysine also induced low levels of metamorphosis at 1 mM (38% ± 13%), but was toxic at concentrations greater than 1 mM.

The responses of the larvae to isoleucine (10 μM) in combination with serine (10 μM, 50% ± 25%) or histidine (10 μM, 58% ± 20%) were not significantly different than the response of the larvae to isoleucine alone (64% ± 18%). Isoleucine (10 μM) in combination with lysine (10 μM) was toxic; all larvae in this treatment appeared to undergo metamorphosis and then die. The response to a combination of isoleucine and valine (each at 50 μM, 27% ± 4.2%) was significantly lower than the response to valine alone (55% ± 7.3%), but not significantly different than the response to isoleucine alone (23% ± 11%).

Metamorphosis assays with acetylsalicylic acid

The response of the larvae to the algal extract was significantly decreased by 1 mM acetylsalicylic acid (Fig. 3). Acetylsalicylic acid concentrations of 0.1 mM and 0.5 mM had no effect either alone or in combination with the extract, and at 5 mM it was toxic alone or in combination with the extract. Acetylsalicylic acid at 1 mM also significantly decreased the responses to isoleucine and valine (Fig. 4). Salicylic acid, unlike acetylsalicylic acid, had no effect on the larval response to *L. poitei* extract (Fig. 5). As discussed above, valine induced significant levels of conch larval metamorphosis, but *N*-acetyl-L-valine had no effect on larval metamorphosis (Fig. 6). Treat-

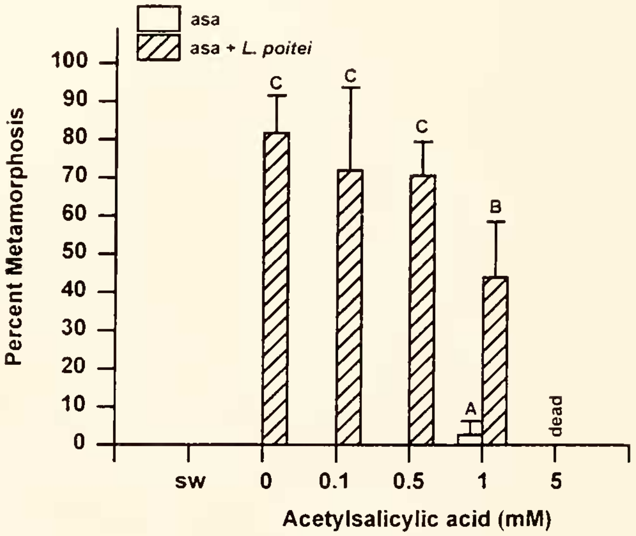

Figure 3. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), acetylsalicylic acid (asa, 0–5 mM) alone, and to asa in combination with an extract of *Laurencia poitei* (20 μl extract/ml seawater). Points are means ± SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$. Dead indicates all larvae in these treatments were dead at $t = 24$ h.

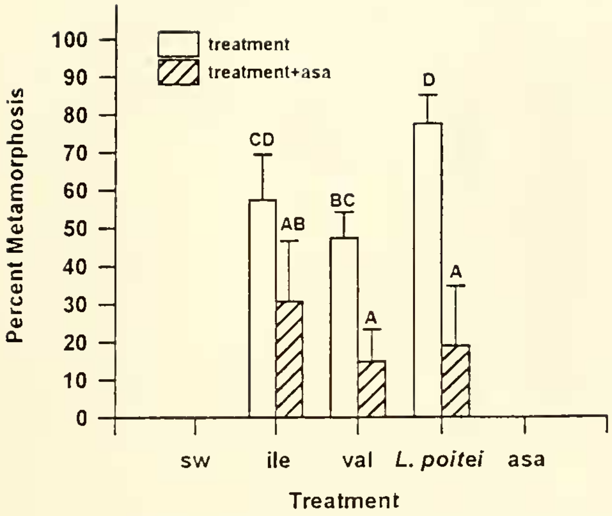

Figure 4. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), isoleucine (ile, 100 μM), valine (val, 100 μM), and an extract of *Laurencia poitei* (20 μl extract/ml seawater) alone, and in combination with acetylsalicylic acid (asa, 1 mM). Asa (1 mM) alone is also shown. Points are means ± SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

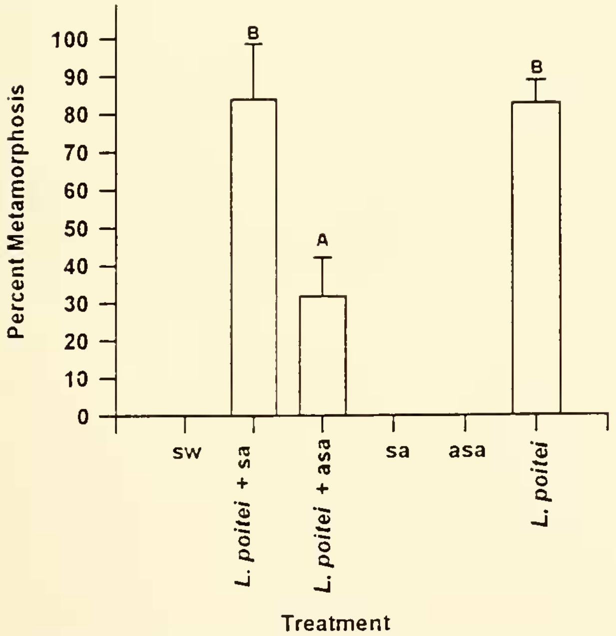

Figure 5. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), an extract of *Laurencia poitei* (20 μl extract/ml seawater) in combination with salicylic acid (sa, 1 mM) or acetylsalicylic acid (asa, 1 mM). Sa, asa, and *L. poitei* extract presented alone are also shown. Points are means ± SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

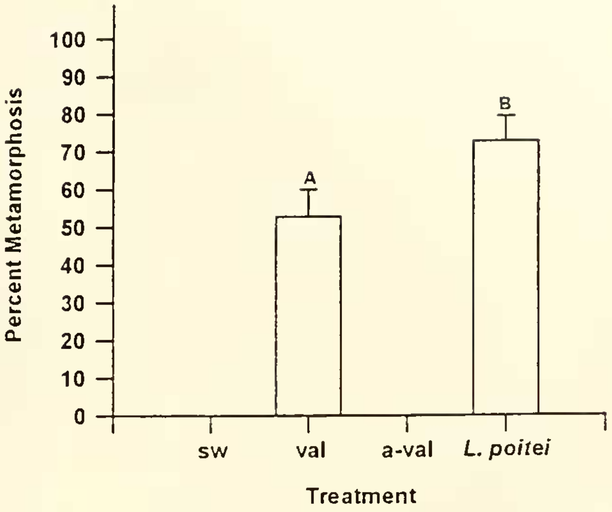

Figure 6. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), valine (val, 100 μM), *N*-acetyl-L-valine (a-val, 100 μM), and an extract of *Laurencia poitei* (20 μl extract/ml seawater). Points are means ± SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

ment of larvae with isoleucine, valine, or algal extract plus acetylsalicylic acid, followed by reexposure to the appropriate cue in the absence of acetylsalicylic acid, induced levels of metamorphosis equal to or greater than those induced by treatment with the cues only. Moreover, the levels of metamorphosis induced by reexposure were significantly higher than those induced by the same cues in combination with acetylsalicylic acid and without retreatment (Fig. 7). In this experiment, the response to isoleucine alone was anomalously low.

IBMX had no effect when presented by itself or in combination with acetylsalicylic acid, but it reduced the effects of acetylsalicylic acid on the metamorphosis induced by the algal extract (Fig. 8). The response to the algal extract plus acetylsalicylic acid (26% ± 12%) was significantly lower than the response to extract alone (88% ± 9.1%). In the presence of IBMX, however, the response to extract plus acetylsalicylic acid (72% ± 7.4%) was not significantly lower than that to the extract alone. Fewer larvae were attached and crawling in the IBMX combination treatment than in the treatment with only algal extract.

Metamorphosis assays with ions and neuroactive compounds

Elevations of Ca^{2+}, Na^{+}, and Mg^{2+} concentrations (60 mM) over ambient seawater levels had no significant effect on metamorphosis. An increase in the concentration of K^{+} to 20 mM over ambient, however, induced signifi-

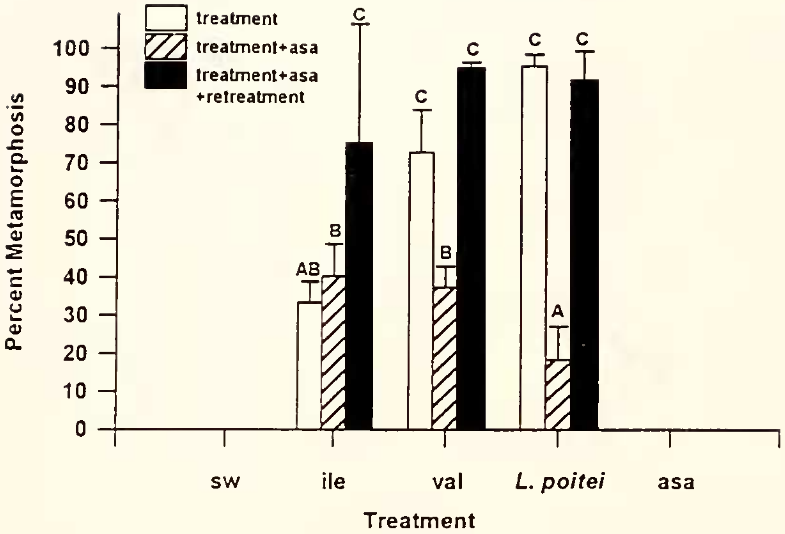

Figure 7. Mean percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), isoleucine (ile, 100 μM), valine (val, 100 μM), and an extract of *Laurencia poitei* (20 μl extract/ml seawater) alone, and in combination with acetylsalicylic acid (asa, 1 mM) with and without reexposure to the appropriate cue. Asa (1 mM) alone is also shown. In this experiment, the response to isoleucine is anomalously low. Points are means ± SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

cant levels of metamorphosis, although, as with other cues, the percentage varied among batches of larvae (Boettcher and Targett, 1996). Elevations in K^+ concentrations between 20 and 22 mM over ambient induced levels of metamorphosis equivalent to those induced by the algal extract. Concentrations ≤ 17 mM or ≥ 24 mM over ambient induced significantly lower levels of metamorphosis, and concentrations lower than 10 mM over ambient had no effect on metamorphosis.

DOPA and EP at 1 μM had no effect on metamorphosis. However, at 10 μM, DOPA induced significant levels of metamorphosis (87% ± 14%), and at 100 μM it had toxic effects. GABA induced significant levels of metamorphosis, but only at concentrations $\geq$ 5 mM (Fig. 9). The levels induced by GABA approached, but never were equal to, those induced by the algal extract. As reported previously, GABA at 1 and 100 μM had no significant effect on larval metamorphosis (Boettcher and Targett, 1996). Hydrogen peroxide at 50 and 100 μM induced levels of metamorphosis (54% ± 16% and 96% ± 4.0% respectively) equal to or greater than those induced by the algal extract (61% ± 5.6% and 93% ± 12%).

TEA at 100 and 500 μM had no significant effect on induction of metamorphosis caused by elevated K^+ concentrations (20 mM), hydrogen peroxide (50 μM), or the extract (20 μl extract/ml seawater), and no significant effect when presented alone (Fig. 10). SITS at 10 μM also had no effect on the above inducers; but at 50 μM, it significantly decreased the response to hydrogen peroxide and to elevated K^+ concentrations. SITS had no effect when presented alone (Fig. 10).

Vanadate (1 mM) induced levels of metamorphosis (53% ± 8.3%) equal to those induced by hydrogen peroxide (32% ± 33%) and *L. poitei* extract (56% ± 20%); but at 2 mM it was toxic to the larvae. Unlike its effects on the induction of metamorphosis by the algal extract, isoleucine, and valine, acetylsalicylic acid had no significant effect on the response of the larvae to hydrogen peroxide. Bovine catalase, however, totally blocked the larval response to hydrogen peroxide, while having no effect on the response to the extract.

Discussion

The natural inducer of larval metamorphosis in queen conch is a water-soluble cue associated with species of red algae, including *L. poitei*, commonly found in conch nursery grounds (Davis and Stoner, 1994; Boettcher and Targett, 1996). Larval responses to this cue are dependent on both concentration and exposure time, with the initiation of metamorphosis occurring within 10 min of treatment. The free amino acids isoleucine and valine, elevations in external concentrations of K^+, the neurotransmitters DOPA and GABA, as well as hydrogen peroxide and vanadate also induce larval metamorphosis.

Isoleucine and valine induce behavioral and morphogenic responses that mimic the effects of the natural inducer of conch metamorphosis. Valine and isoleucine are

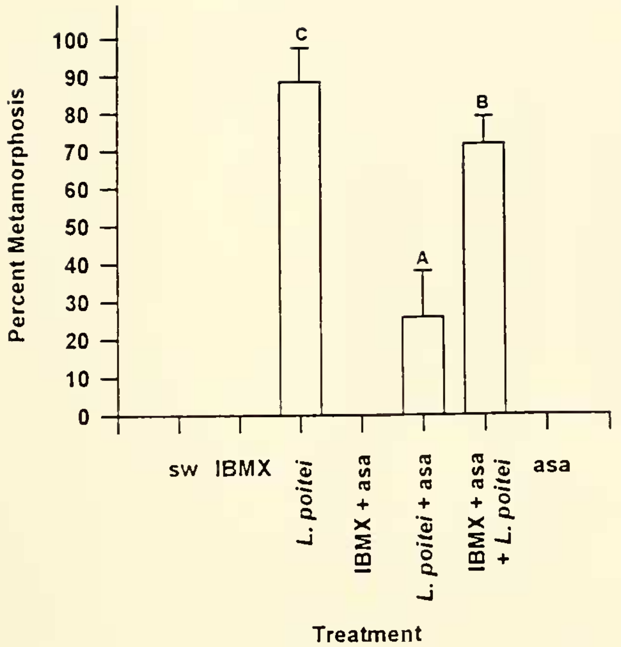

Figure 8. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), isobutyl methyl xanthine (IBMX, 0.1 mM) and an extract of Laurencia poitei (20 μl extract/ml seawater) alone, in combination with acetylsalicylic acid (asa, 1 mM), and in combination with one another and asa (1 mM). Asa (1 mM) alone is also shown. Points are means ± SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

similar in that they have neutral, hydrophobic, branched side chains that differ only in the length of one of the branches (Fig. 11). In leucine, the methyl group is displaced, and though this amino acid induces significant levels of metamorphosis, it does not induce normal larval behavior, and it is toxic at concentrations ≥ 100 μM. Several other amino acids also induce partial metamorphosis, but not normal larval behavior (Table II). Glutamic acid, glycine, and threonine, although found at higher concentrations than valine, isoleucine, or leucine in the <1 kDa component of hydrolyzed algal extract, induced only low levels or no metamorphosis.

The responses of conch larvae to the algal extract, valine, and isoleucine are blocked by acetylsalicylic acid (Fig. 4). In other systems (Hara, 1977), alterations of the α-amino or α-carboxyl group on amino acids through acetylation, methylation, or esterification block the activity of amino acids. Hara's results (1977) coupled with the results of our experiments on the effects of *N*-acetyl-L-valine suggest that acetylsalicylic acid may be modifying sites on either the inducer (*L. poitei* extract, valine, or isoleucine) or the larval receptors, possibly through acetylation. Acetylsalicylic acid, however, may also affect transduction of the metamorphic signal, since it indirectly affects levels of cAMP in cells (Hecker *et al.*, 1995; Payan and Katzung, 1995). Although the cAMP phosphodiesterase inhibitor IBMX does not, by itself, have an effect on conch larval metamorphosis, it does alleviate the negative effects of acetylsalicylic acid on metamorphosis (Fig. 8). Therefore, the ability of acetylsalicylic acid to modulate

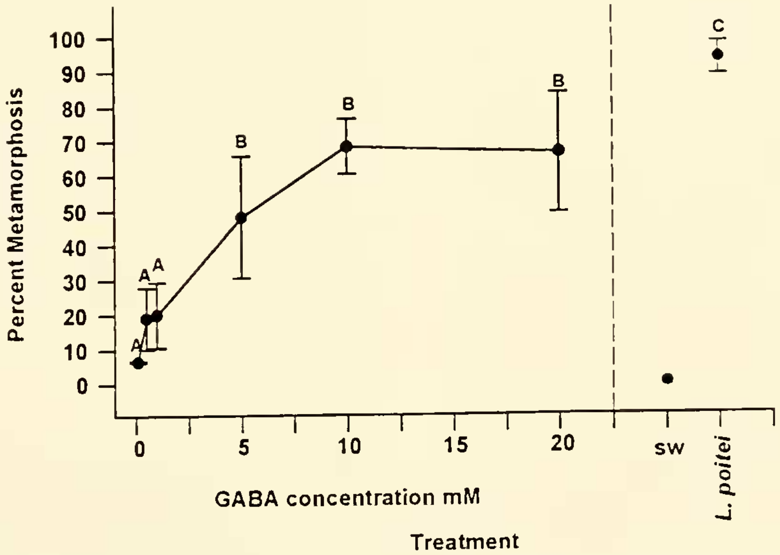

Figure 9. Percent metamorphosis of queen conch larvae in response to specific concentrations of γ-aminobutyric acid (GABA). Seawater only (sw, a negative control) and an extract of *Laurencia poitei* (20 μl extract/ml seawater) are also shown. Points are means ± SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

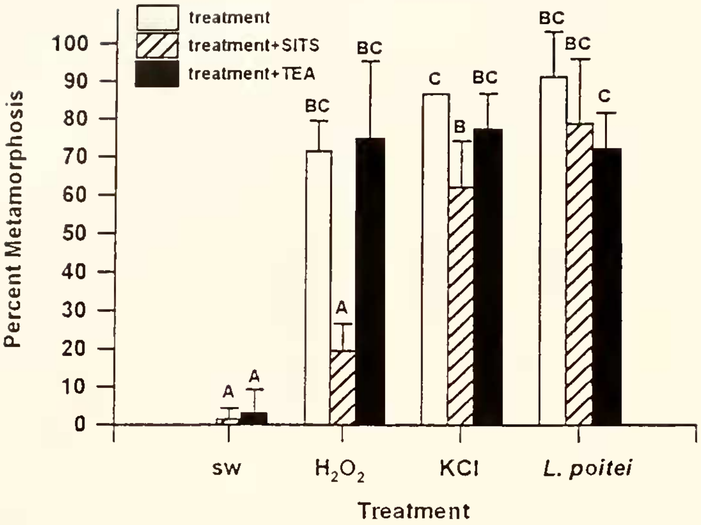

Figure 10. Percent metamorphosis of queen conch larvae in response to seawater only (sw, a negative control), hydrogen peroxide (H_2O_2, 50 μM), increased KCl (20 mM) and an extract of *Laurencia poitei* (20 μl extract/ml seawater) alone and in combination with 4-acetamino-4'isothiostilbene-2,2'disulfonic acid (50 μM, SITS) or tetraethylammonium chloride (500 μM, TEA). Points are means ± SD; $n = 5$. Data points with the same letter above the error bar are not significantly different at $P \leq 0.05$.

cAMP levels during conch metamorphosis cannot be disregarded. However, since salicylic acid (a compound that, like acetylsalicylic acid, can affect cAMP levels) has no effect on conch larval metamorphosis, and since metamorphosis induced by hydrogen peroxide is unaffected by acetylsalicylic acid, it is unlikely that the effects of acetylsalicylic acid are due to its influence on the cAMP second messenger system.

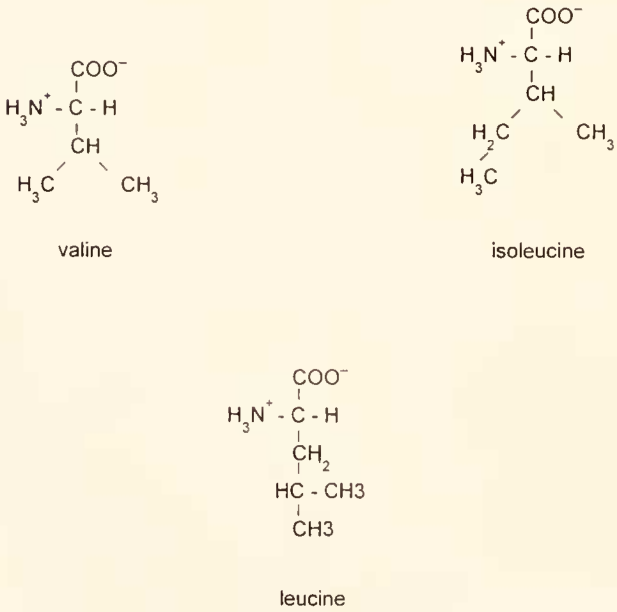

Figure 11. Structure of the amino acids valine, isoleucine, and leucine.

The responses of conch larvae to hydrogen peroxide, DOPA, vanadate, and GABA are consistent with the known roles of second messenger pathways in settlement and metamorphosis, as well as with the known activities of these compounds in other systems. Models for processes controlling the transduction of metamorphogenic signals in other marine larval systems have drawn on those developed for olfactory responses, involving primarily the adenylate cyclase (AC)/cAMP pathway, the phospholipase C (PLC)/inositol triphosphate (IP_3) pathway, or both (Leitz and Müller, 1987; Freeman and Ridgeway, 1990; Morse, 1990; Rittschof *et al.*, 1991; Anholt, 1992; Fadool and Ache, 1992; Michel and Ache, 1992; Clare *et al.*, 1995; Brunet *et al.*, 1996).

Hydrogen peroxide, vanadate, and DOPA all induce complete larval metamorphosis in the queen conch. The response of conch larvae to DOPA appears to be related to the production of hydrogen peroxide in the breakdown of this catecholamine rather than to any direct effect of DOPA itself; no response is initiated in the DOPA treatments until peroxide concentrations reach levels that alone induce metamorphosis (AAB, pers. obs.). This is similar to the response of *Phestilla sibogae* larvae to DOPA and hydrogen peroxide (Pires and Hadfield, 1991). Although hydrogen peroxide induces complete metamorphosis in the conch, only partial metamorphosis is included in *P. sibogae* (Pires and Hadfield, 1991). In other systems, hydrogen peroxide and vanadate have been shown to directly or indirectly activate Ca^{2+} release channels via oxidation of thiol groups, stimulate phospholipase C, and increase protein tyrosine phosphorylation (Paris and Pouysségur, 1987; Ranjan and Goetz, 1990; Leitz and Wirth, 1991; Pires and Hadfield, 1991; Favero *et al.*, 1995). In addition, it has recently been hypothesized that hydrogen peroxide, like nitric oxide, may itself be an intracellular second messenger (Dong, 1995; Sundaresan *et al.*, 1995). One or several of these mechanisms may be responsible for the activities of hydrogen peroxide, DOPA, and vanadate in conch larval metamorphosis, and all suggest that these compounds influence metamorphosis by modulating second messenger pathways.

The types of chemical cues involved in conch metamorphosis and the mechanisms controlling it share general features with chemoreception in adult aquatic invertebrates (Burke, 1983; Baxter and Morse, 1987; Arkett *et al.*, 1989; Carr *et al.*, 1989; Bonar *et al.*, 1990; Freeman and Ridgway, 1990; Morse, 1990; Pawlik, 1990; Leitz, 1993; Leitz *et al.*, 1994). The red algal cues, like those that induce olfactory responses in other marine organisms, are of low molecular weight and soluble in water. It appears that the molecules of the algal cue bind to sites that also recognize and bind particular amino acids (*i.e.*, valine and isoleucine). The results of the experiments with vanadate and hydrogen peroxide suggest that metamorphosis induced by the natural cue may be triggered through a second messenger pathway. As with other marine larvae, elevated K^+ concentrations (above ambient seawater) can directly activate the metamorphosis process, presumably by depolarizing sensory cells. Studies focusing on further characterization of the natural inducer, and on the potential involvement of the PLC/DAG/IP_3 and AC/cAMP second messenger systems in conch metamorphosis, will deepen our understanding of the metamorphic process in conch and in other marine invertebrates. Electrophysiological studies directly testing the effects of morphogens on potential larval chemoreceptors would contribute further to the understanding of how metamorphosis is induced and how the signals are transduced, and would also aid in the identification of the active components of the cues.

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